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Influence of the Extraction Process on the Characteristics of Romanian Mountain Walnut Oil

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Abstract

The objective of this work is to extract walnut oil using various processes in order to compare the influence on the nature of the components extracted, and thus identify the areas of potential use. We carried out the extractions by mechanical process, thanks to a press in reduced model provided with a worm. We obtained cold extracted oil whose characteristics slightly diverge from extra virgin oil found in shops in Romania, but its composition is similar. We were also able to extract by chemical process using two methods, Folch and Soxhlet. Commercially available table walnut oils are only cold extracted to avoid the presence of solvents. Those are difficult to remove and strongly oxidize the oil. Currently, consumers appreciate walnut oil for its taste and nutritional qualities. In nutrition, this oil is put forward for its composition rich in polyunsaturated fatty acids, which are needed for human body. Food supplements made from walnut oil are available today. For the moment, this is the only use of walnut oil. Indeed, there are some studies on other fields of application, but they remain in the field of research and nothing has yet been commercialized. In this present study, we compared the chemical and physical properties of cold-extracted oil with the solvent extraction of walnut kernel originating from the mountain region of Rumania. The cold extracted oil has a high content of polyunsaturated fatty acids (63%) and monounsaturated fatty acids (30%), a very low level of saturated fatty acid (7%) and no content of linolenic acid. The Soxhlet and Folch methods produced slightly different

oils with increased amounts of minor components, which changes their characteristic. Even when solvent-extracted oils do not meet the standard criteria imposed by the Codex Alimentarius, they offer a possible use in the fields of food, cosmetics industries and biomedicine.

Keywords

Walnut Oil, Extraction, Composition, Romanian Mountain

1. Introduction

The walnut is the fruit of a tree called the walnut tree (*Juglans regia* L.), which has been imported from Asia. Today, this tree is found almost everywhere on the planet. The countries where the walnut is most cultivated are China and the United States. In Europe, the two main producers are France and Romania. Walnuts are an expensive commodity; this is why production is lower than for other oleaginous fruits used to produce oil, such as palm or soybeans.

Humans have been eating and using nuts since immemorial times. Indeed, in addition to eating walnut kernels, men used walnut oil to create natural remedies and skin care products [1] [2]. In folk medicine, walnut oil was used and known for its anti-diarrheal and antiseptic properties, and has beneficial effects against oxidative stress mediated diseases such as cardiovascular diseases and cancers [3].

In 2022/2023, the global walnut production reached about 1.2 million tons, which is the highest volume in a decade and more than twice the crop volume in 2012/2013. China was the worldwide walnut leader, by producing 616 thousand tons of kernel basis (53%). The fourth largest producer, a European one (Ukraine), yielded for its part 34.5 tons (3%) [4]. Walnut is sold to consumers both as kernels and as oil. This oil is appreciated as table oil for its sweet and typical taste. Commercial walnut oils are often found under the name virgin or extra virgin oils (corresponding to the oil extracted by PITEBA). This denomination indicates that these oils were cold-pressed extracted. Refined nut oils are rare or even non-existent. There are also butters made from a mixture of vegetable fats, called margarine, and a few brands have released a new line with nut oil in their recipe [5].

The oil is also recognized for its nutritional values since it contains a significant amount of essential fatty acids that our body uses to meet many of its needs [2]. Fatty acids play a role in energy storage and in the structuring of cell membranes. The essential fatty acids present in walnut oil are linolenic acid, known to be the precursor of omega 3 and linoleic acid, known to be the precursor of omega 6 fatty acids [1] [6]-[8]. People are forced to bring these molecules through food because bodies are unable to synthesize them. There is an optimal quantitative relationship between these fatty acids, which is important to respect; otherwise, the human body cannot properly use the two fatty acids [9]. The ratio of omega 6 out of 3 must be less than 5 out of 1. Walnut oil is richer in omega 6 than in omega 3. Food

supplements created based on walnut oil [10] are therefore able to respect this ratio. If this proportion is not respected, in the long term it can cause problems in the nervous and inflammatory system. Omega 3 has important roles in the development and maintenance of the nervous and ocular systems. On the other hand, omega 6 is involved in the production of cell membranes, in immune defences, in the inflammatory response, as well as in reproduction [2] [11].

1.1. Walnut Oil

Nowadays, several studies are made regarding the use of walnut oil components in health care [2] and biodiesel [12], for example. However, these studies are on research stages, and today, the field in which walnut oil is the most used is in the food industry. Indeed, walnuts and walnut oil are valued for their typical taste and are sold as is or as table oil. In the food industry, walnut oil is most often extracted by mechanical and cold press processes, since oils with the mention "virgin" or "extra virgin" are only found.

1.2. Oil Triglycerides

The oil is composed of 95% to 99% of triglycerides [13] [14], 90% to 95% represents the percentage of fatty acids and 3% to 5% represents the percentage of glycerol in the oil. Triglycerides are considered as neutral lipids or saponified lipids. Fatty acids are composed of a carboxyl group and a more or less long carbon chain (**Figure 1**).

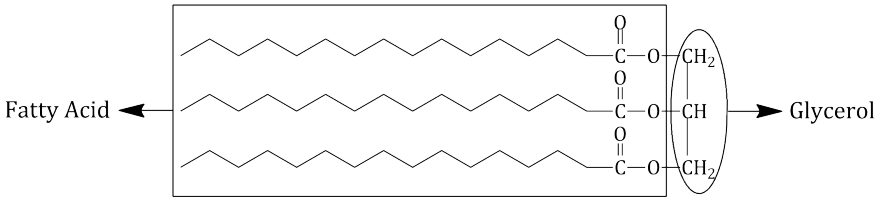


Figure 1. General structure of a triglyceride.

This carbon chain always has an even number of carbons, can be composed of four to twenty-two carbons and can have one or more double bonds. These fatty acids have the characteristic of being volatile. For this reason, they are the ones we analyse in lipids and not triglycerides, which are too large non-volatile molecules. The main types of fatty acids that we find in walnut oil are listed in **Table 1**.

Table 1. Main fatty acids in walnut oil.

Satured	Palmitic Acid C16:0 (5%)
	Stearic Acid C18:0 (3%)
Mono-unsatured	Oleic Acid C18:1 (15% - 23%)
Poly-unsatured	Linoleic Acid C18:2 (57% - 62%)
	Linolenic Acid C18:3 (12% - 15%)

There are also other fatty acids (Table 2) but in minor quantities compared to those seen above.

Table 2. Other fatty acids in walnut oil.

Satured	Heptadecanoic Acid C17:0
	Arachidic Acid C20:0
Mono-unsatured	Hexadecanoic Acid C16:1
	Eicosenoic Acid C20:1
Poly-unsatured	Heptadecadienoic Acid C17:2

1.3. Other Components

The other components of the oil represent 1% to 5% in weight percentage. We find polar lipids, which represent 0.1% to 0.2% of the oil. They are in minor quantities and do not play a characteristic role in the oil. The 0.1% to 3% remaining concern the unsaponifiable constituents, such as tocopherols and sterols.

Tocopherols are molecules found in walnut oil. These molecules are part of the group of vitamins E, antioxidant molecules that trap free radicals responsible for the oxidation of nuts and oil. Tocopherols also allow better conservation of the nuts and therefore of the oil. There are four kinds of tocopherols: alpha, beta, gamma and delta. Tocopherols are present in small quantities, on the order of micrograms, but they are sufficient to play the role of antioxidant in lipid environments [15]. Vitamin E is sold to meet skin and hair care needs, more particularly against aging of the skin [16] [17]. Vitamin K, or the phylloquinone molecule, although in small quantities in walnut oil, is often put forward because this vitamin allows good healing. It is sold as a medicine in the treatment of haemorrhages caused by a deficiency of vitamin K [18] [19]. However, these last two molecules are not necessarily extracted and made from walnut oil. Other components of the sterol family are present in walnut oil. They are found in smaller quantities. These are only certain sterols, *i.e.*, beta-sitosterol, campesterol and Δ5-avenasterol. These sterols, from a nutritional point of view, are very good for lowering cholesterol. Cholesterol is an important molecule that is synthesized by our bodies and provided by our food. As a result, sometimes too much cholesterol is present in our blood. The sterols in walnut oil help reduce this intake. The human intestines do not absorb them, so their role is to partially block the absorption of cholesterol during digestion and regulate cholesterol intake [20].

Extraction of oil and other valuable components is strongly dependent on the method used for the extraction. Chloroform/methanol and hexane are generally considered as the best extraction solvents for most of the edible oils on a laboratory-scale [11]. The solvent extraction method must be chosen according to the oil characteristics and may induce partial alteration of most of the minor ingredients that have many antioxidative, functional and pro-oxidative effects. However, the remnant solvent makes this extraction unsuitable for human dietary consumption. Cold press extraction is the commercial method applicable to extract

oil from oilseeds for the food industry, but may not be the best process for other purposes such as cosmetic or pharmaceutical uses [21]. The aim of this study was to compare different extraction methods with respect to the oil content, fatty acid profile, physicochemical characteristics, and antioxidant activities of the extracted oils from walnut kernel. Additionally, we reported here the physical and chemical characteristics of walnut oil extracted from an unusual source, the mountain region of Rumania.

2. Materials & Methods

2.1. Oil Extraction

2.1.1. PITEBA Press

The nuts are dried after the harvest and before commercial trade. The moisture content of the nuts, when they arrive at the laboratory, is therefore already lower and allows the oil to be extracted directly.

The procedure for extraction with PITEBA [22] is simple. The extraction was carried out at a maximum of 40˚C, for being considered extraction in cold. Then, diced kernels are added into the funnel by turning the crank so that the worm extracts the oil. The residue from the nuts is called "pulp" and comes out in the form of granules through the holes in the cap located at the end of the worm. This pulp can later be sold as animal feed. The oil obtained contains impurities, which are eliminated by filtration using a Buchner apparatus. After filtration, the oil is yellow in colour and not low in impurities. It is stored at 5˚C and protected from light.

2.1.2. Soxhlet Method

The nuts were grounded into fine pieces to facilitate extraction. 40 g of crushed nuts were placed in a thick paper cartridge in the Soxhlet apparatus. 200 mL of hexane were added into a round bottom flask and heated to 30˚C for 5 h and allowed to cool for 30 min before recovering the oiled solution plus hexane from the flask. A second test was performed with a larger mass of nuts: 60 g of crushed nuts were added for 300 mL of hexane. After extraction, the flask containing the oiled and hexane solution, was passed through the vacuum evaporator in order to remove the hexane and only recover the oil. Hexane has a boiling temperature of 69˚C, while that of oil is ~200˚C. The flask containing the solution of hexane or oil were immersed in a water bath at 40˚C.

2.1.3. Folch Method

50 g of chopped walnut kernels were mixed with 180 mL of demineralized water, 100 mL of methanol and 200 mL of chloroform. This solution was mixed and centrifuged at 5000 rpm for 2 min. 100 mL of chloroform were further added and the mixture was centrifuged at 7000 rpm for 10 min. The solution was filtered using a Buchner apparatus for recovering the nut residues. 100 mL of chloroform were further added to the residues to re-extract the remaining oil and then filtered again. Following this procedure, two filtered solutions were brought together and placed in a separatory funnel for 1 h in order to recover the lower phase in which

the oil and the chloroform are contained. This solution was concentrated under reduced pressure to remove chloroform and methanol.

2.2. Oil Analysis

2.2.1. Colour

The determination of the colour is carried out visually.

2.2.2. Clarity

The determination of the clarity is carried out visually.

2.2.3. Refraction Indice

The values of the refractive index were determined with the Abbe refractometer.

2.2.4. Density

A pycnometer was used for density measurements [23]. Calibration was attained by weighing the empty and demineralized water-filled pycnometer at 25˚C. The same procedure is carried out with the oil extracted by PITEBA, Soxhlet, for commercial extra virgin oil and for oil extracted by the Folch method. Once all the masses have been noted, the density of each of the oils was calculated.

2.2.5. Viscosity

The study of rheology is important to characterizing the oils, as it is a criterion of oil quality. This analysis was carried out using a rotary viscometer. Its operation is based on the torsional resistance of a liquid relative to the rotation of a rod of known characteristic and submerged in this liquid. The deflection angle of the rod, which is measured electronically, gives the measure of the torsional force.

The rheological characterization of the oils was carried out under thermostatic conditions (temperature range from 25˚C to 45˚C), using a rotation viscometer Rheotest-2. The device allows the measurement of the torsion moment resulting from the resistance of the ring-shaped substance layer placed between a fixed cylinder and a rotating one with known revolution. The torsion moment is correlated with the shear stress (τ). The revolution and the ring-shaped layer thickness determine the shear rate ($\dot{\gamma}$). The flow curves $\tau = f(\dot{\gamma})$ of the oil samples were plotted with the shear rate in the range 3 ÷ 1312 s^{-1}.

The calculations carried out in the viscometer from measurements of the torsional force, the speed of the axis and its characteristics, give a direct reading of the viscosity in Pascal second (Pa.s), knowing that 1 millipascal second (mPa.s) is equivalent to 1 centipoise (cP).

2.2.6. Humidity Percentage

The moisture content of the nuts was determined with a thermo-balance (MA X2.A moisture analyzer—Radwag Balances and Scales). The heating program was 30 min at 100˚C. The results were computed during the entire heating period.

2.2.7. Acid Index

The amount of free fatty acid in each sample was estimated by titration and

calculation of the acid index. During titration, sodium hydroxide will react with the free fatty acids that are possibly present in the oil and cause a change in pH, which will be detected by a colour change of phenolphthalein. The pH is assumed to be slightly acidic because of the free fatty acids. Therefore, before titration the solution is colourless and when the NaOH reacts with them, the pH becomes basic and the phenolphthalein present in the medium will then turn pink.

The titration of the oil was determined by the amount of titrating solution that was necessary to neutralize the solution. The titrating solution contains sodium hydroxide. To determine the concentration of NaOH, a titration with sulphuric acid was carried out, in the presence of the pH indicator phenolphthalein. The equation of the reaction is:

$$2NaOH + H_2SO_4 \rightarrow Na_2SO_4 + 2H_2O$$

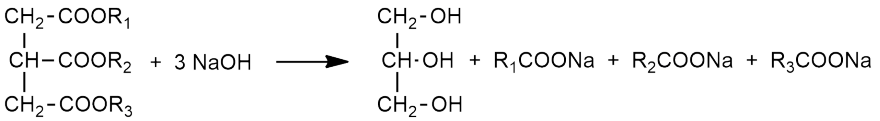
From the volume of sulphuric acid, which reacted with the sodium hydroxide present in the medium, a correction factor for the concentration was calculated and used for calculating the acidity index.

The titrated solution contains 3 g to 5 g of oil, 1 to 2 drops of phenolphthalein and 20 mL of a mixture of ethyl alcohol and ethyl ether used to dissolve the oil with the titrating solution. The titration reaction is 1 to 1 molar stoichiometry for sodium hydroxide and free fatty acid:

$$RCOOH + NaOH \rightarrow RCOONa + H_2O$$

2.2.8. Saponification Index

Saponification Index was determinate by indirect or reverse titration. 0.3 to 0.5 g of oil was added to a solution of 30 mL of sodium hydroxide and 20 mL of a mixture of 1:1 ethyl ether/ethyl alcohol. The latter allows dissolving the oil for a complete reaction with alkali. This solution was placed on a reflux heating assembly. The solution was heated to ~150˚C for one hour from boiling. The equation of the reaction is:



Once the saponification stage has been completed, the solution is titrated, in the presence of phenolphthalein, with hydrochloric acid at a concentration of 0.5 mol/L. The solution, in presence of phenolphthalein, was pink. Along the titration, all the excess sodium hydroxide molecules will react with hydrochloric acid, and the solution becomes colourless. The equation for the reverse titration reaction is:

$$HCl + NaOH \rightarrow NaCl + H_2O$$

2.2.9. Iodine Index

The Iodine Index evaluated the unsaturation level of the oil samples. 0.1 g of walnut oil was weighted and put in an Erlenmeyer flask with 5 mL of toluene to facilitate its solubilisation with solvents. 15 mL of Hanus reagent is then added, which

contains iod-monobromide. After stirring, the solution is placed for 15 minutes in dark. The iodide ions will react with the unsaturation's of the triglycerides acyl chains. Then, 10 mL of potassium iodide, 30 mL of water and 2 to 3 drops of starch paste were added under stirring. A black colour was observed in the organic phase and a blue one in the aqueous phase.

An indirect titration of the solution was then carried out with a 0.1 mol/L sodium thiosulfate solution. The titration is completed when the solution becomes colourless. The equation for the titration reaction is:

$$2Na_2S_2O_3 + I_2 \rightarrow 2NaI + Na_2S_4O_6$$

2.2.10. Peroxide Index

Approximately 5 g of walnut oil was weighted, and a mixture of 18 mL acetic acid and 12 mL chloroform was added to allow better solubilisation in the solvent. Then, a potassium iodide solution and 30 mL of demineralized water were added. After homogenization, this solution is titrated with sodium thiosulfate at a concentration of 0.01 mol/L, until the solution turns yellow. 2 mL of starch were added to test if iodine remains, which will be evidenced by the occurrence of a blue coloured complex. The titration was completed until the solution becomes colourless.

2.2.11. Mineral Composition

The oil samples were calcined at 550˚C for 5 h to remove the organic matter. The resulting ash was dissolved with 10 mL of hydrochloric acid and 5 mL of nitric acid. The ash solution was further dissolved in 50 ml of distilled water; the sample has been previously filtered and washed with distilled water. The analysis of the components was carried out by atomic absorbing spectrometry, using the Varian atomic adsorption flame DD 280 FS.

2.2.12. UV-Visible Spectroscopy

Oil samples were prepared by dilution 1/10 with a cyclohexanol solution. UV-VIS spectra were recorded on an Agilent 60 UV-VIS spectrophotometer at 20˚C.

2.2.13. Infrared Spectroscopy

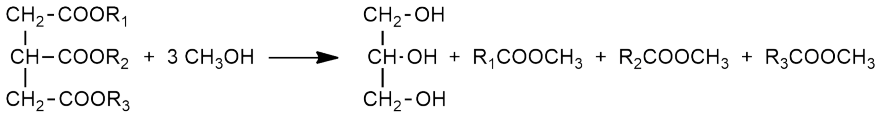
FT-IR spectra were recorded with a Bruker Vertex 70 spectrometer (Bruker Daltonik GmbH, Germany) equipped with a Platinium ATR spectrometer, Bruker Diamond type A225/Q.I.

2.3. Fatty Acids Analysis

2.3.1. Derivatization Assay

The aim of derivatization procedure is to transform triglycerides into more volatile fatty acid methyl esters. It is achieved by a transesterification of the oil with methanol in a basic medium. Adding 0.15 g to 0.2 g of oil to a round-bottom flask with a volume of 50 mL performed saponification. 5 mL of a mixture of sodium hydroxide (10%) and methanol were added thereto. This solution was refluxed 15

min from the boiling of the solution. Methylation consists of stabilizing the fatty acids by adding a methyl group at the end of the acyl chain. 5 mL of boron trifluoride in methanol (BF_3-CH_3OH) were added to the solution and the mixture was allowed to proceed 5 min from boiling. This boron trifluoride product served as a catalyst for the reaction. 10 mL of hexane were further added, and 1 min was allowed to act at the boiling point. This last step was performed for favouring the extraction of fatty acids from the rest of the solvents by promoting phase separation. Once the solution has cooled, the upper organic phase enriched in hexane and the methylated fatty acids was recovered, and then transferred to a small bottle and stored at 5˚C until GC analysis. The simplified transesterification reaction is:



2.3.2. Gas Chromatography—Mass Spectrometry

Chromatograms and mass spectra were obtained by using the GC-MS thermo scientific TRACE 1310 ITQ 1100 Ion Trap MS system. The column used was TG-SMS, 30 cm length and 0.25 mm diameter; the thickness of the layer of active substance was 0.25 µm. The CPG heating program was settled in 150˚C to 240˚C, with a temperature increase of 2˚C/min, the temperature stabilizes at 240˚C for 0.5 minutes, then it increases to 300˚C at a 7˚C/min rate until the temperature stabilizes at 300˚C for 2 min. The temperature in the injector was 250˚C, the flow rate of the carrier helium gas, was 1 mL/min.

Parameters of mass spectroscopy: the fraction which connects the CPG to the SM was kept at 310˚C and the interval in which the ratio between the mass and the load of the analytes is determined was 35 to 700 m/c. The internal standard was hexadecane.

3. Results & Discussion

3.1. Oil Extraction

3.1.1. Mechanical Extraction

Several methods of extracting oil were carried out for the present study. The first was mechanical extraction:

- By hydraulic press: the nuts are crushed into fine pieces and then cold pressed by pressing.
- By mechanical press: the nuts are cold pressed by an endless screw.
- By centrifugation: the oil and the dry matter of the nuts are separated by a process which accentuates the natural phenomenon of separation by difference in weight.

For reasons of practicality and price, it is the mechanical press with worm, which was chosen within the scope of our study. The chosen press is called PITEBA.

PITEBA Press

The tests carried out by mechanical extraction were not profitable (Table 3), this can be explained by the fact that the press is rudimentary and many parameters, such as the temperature, the adjustment of the screw and the pressure within the press is not known and cannot be adjusted precisely.

Table 3. PITEBA extraction results.

	Trial 1	Trial 2
Mass pieces of nuts after drying (g)	471.37	381.30
Oil mass obtained (g)	34.65	29.36
Oil percentage obtained (w/w) (%)	7.35	7.70

The percentage of extraction by the PITEBA machine, for these tests, is on average 7.5%. According to the PITEBA website [22] [24] we should theoretically have obtained a return of 47% to 48%.

The advantage of mechanical extraction is not to use any chemicals and therefore does not contain any foreign substance. From an industrial point of view, this is a guarantee of quality. Furthermore, there is no need for a purification step, so it saves time and money. The pulp can also be sold which is an added advantage. However, with this technique there are some losses in oiled since a part remained trapped in the press and another in the pulp.

These mechanical extraction processes are used by industrialists to promote the quality of their product and to be part of a more eco-responsible approach by using processes which do not use chemical products and thus avoid reprocessing of water which is currently expensive to industrialists.

3.1.2. Solvent Extraction

The second extraction process requires solvent, as part of a laboratory extraction there is several suitable methods and for each of them specific solvents are used (Table 4).

Table 4. Solvent extraction methods.

Method	Solvent
Soxhlet	Hexane, petroleum ether
Folch	Methanol, Chloroform
Reflux heating	Ethanol, isopropanol, ethyl acetate, acetone, iso-hexane, n-hexane

Here, the Folch and Soxhlet methods were chosen to carry out the solvent extraction. We did not use the reflux heating method because the most profitable solvents for this method were not available in sufficient quantities in the laboratory.

Soxhlet Method

The Soxhlet method (Table 5) is, therefore, more profitable than the PITEBA extraction method.

Table 5. Soxhlet extraction results.

	Trial 1	Trial 2
Mass of kernels (g)	40	60
Oil mass obtained (g)	23.9	35.8
Yield percentage (w/w) (%)	59.8	59.7

Indeed, according to the website [22] the yield of PITEBA is lower than that of Soxhlet. Regarding the Soxhlet method, it allows the extraction of oil while using little solvent. Furthermore, with this method the solvent is recycled and reused. However, there are still drawbacks to this method, the extraction time is quite long and requires a large amount of water which can cause a significant energy cost. Furthermore, heating can lead to the denaturation of oil components. Purification is also necessary to remove all foreign and inedible substances from the oil.

The Soxhlet method is used only for laboratory purposes. In industry, extraction is done with the same solvent, hexane. The solvent extraction process is used by industry to determine the quantity and not for achieving high-quality oil. The extraction process in industry is based on the same principle of extraction as in the laboratory, but it is adapted to produce a larger quantity of oil. So, the common factor between a laboratory extraction and an industrial extraction is the type of solvent used and the temperature.

Folch Method

This method uses a principle of extraction by cold maceration. The raw material, here the nuts, is mixed with specific solvents to the method: methanol and chloroform (Table 6).

Table 6. Folch extraction results.

	Trial 1	Trial 2
Mass of kernels (g)	50.0	50.1
Oil mass obtained (g)	20.1	20.1
Yield percentage (w/w) (%)	40.2	40.1

The Folch method is less profitable, according to these results, than the method by Soxhlet and PITEBA. This production method is costly because in total we used nearly 1200 mL of chloroform and 200 mL of methanol for 100 g of nuts. These solvents have an impact on the oil that reacts with them and can oxidize faster. Moreover, these products are not reused as with Soxhlet but discarded and implies an expensive treatment. The positive point of this method is that it does not heat the oil and therefore avoids denaturation by heat and has a positive energy balance since it does not use a water circuit to cool or heat.

3.2. Oil Analysis

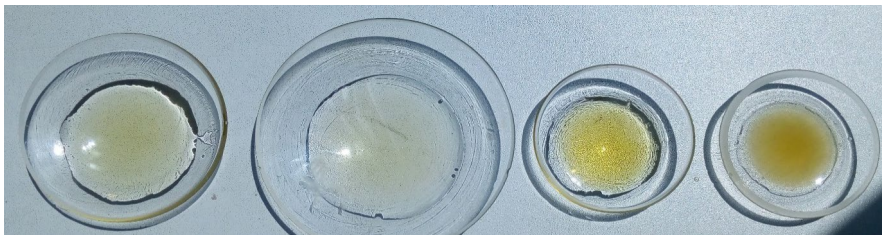
To characterize the oils, analyses were carried out on the three oils extracted

according to the three processes used and by comparison with commercial extra virgin walnut oil. Our oils are extracted from nuts from the mountains of Romania, while commercial oil is extracted from nuts from the plains of Romania [6].

3.2.1. Physicochemical Characterizations

In the oil extracted by Folch we observe residues. This is due to cold maceration, which reduced the matrix of the oil. These impurities, being too small, could not be removed during filtration. Compared to commercial oil, the oil extracted by PITEBA and Soxhlet is pale in colour. In addition, according to the European standard, it is possible to add a precise dose of dye only if it would have lost its colour during the process, except for the label of the commercial oil state that the product has not added dye. This difference can be explained by the different growth origins of the nuts (Table 7).

Table 7. Oil colour comparison.

Oil	PITEBA	Soxhlet	Commercial	Folch
				
Coloration	Pale yellow	Very pale yellow	Yellow	Yellow orange
Clarity	Translucent	Translucent	Translucent	Weak opalescent

The different extraction methods showed no discernible influence or difference on the odour and flavour of the oil.

It is observed that the refractive indices of the oils are roughly similar. The slight difference observed can be explained by the fact that the oil extracted by Soxhlet and Folch uses solvents. This extraction method produces a considerable amount of minor compounds, which changes the percentage of oil composition and, therefore, the refractive index (Table 8).

Table 8. Physicochemical and chemical characterizations of the oils obtained.

Oil	PITEBA	Soxhlet	Commercial	Folch
Refraction index	1.478	1.475	1.478	1.475
Density g/m^3	0.927	0.926	0.913	0.926

The density of oils, in general, is around 0.920 g/cm^3. Our oils have a higher density compared to commercial oil. This result is due to the presence of larger molecules. This evidences that our oils have not undergone the same treatments as industrial oil or have a difference in the source nuts oil content, which creates this little difference.

3.2.2. Viscosity

It is observed (Figure 2) that the oil extracted by PITEBA at room temperature, 25˚C, has a viscosity ~50 centipoises, while the oil extracted by Soxhlet has a viscosity ~33 centipoises. This difference decreases as the temperature increases. Regarding commercial oil, we have a viscosity ~50 centipoise at 25˚C, as for the oil extracted by Folch its viscosity at 25˚C is 5 centipoises (Figure 3).

According to these results, the oil obtained *via* Soxhlet extraction has a lower viscosity than the oil extracted by PITEBA and commercial oil. These last two oils have the same viscosity, so oils extracted by mechanical process offer good viscosity. As for the oil extracted by Folch, it is almost liquid, it is assumed that there are other compounds present in the extract; the oil is therefore probably not pure. Oils extracted by solvent do not allow to obtain a viscosity according to standard procedures.

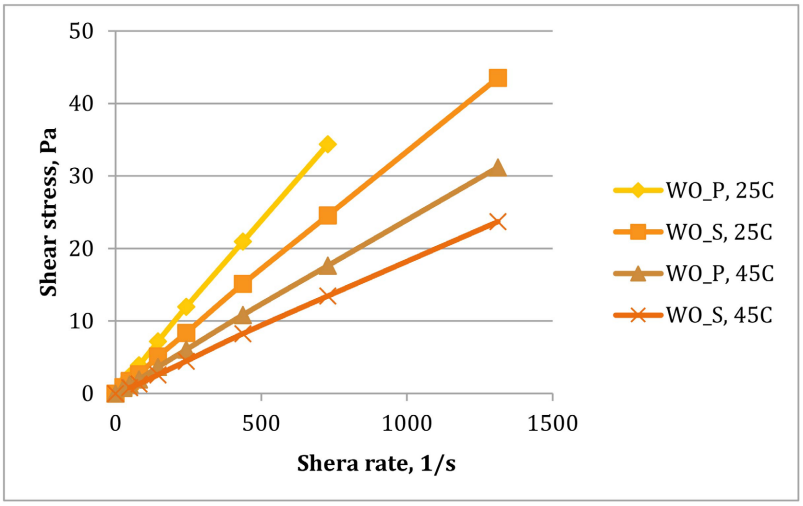


Figure 2. Viscosity of the oils (WO) extracted by PITEBA (P) and Soxhlet (S) performed at different temperature.

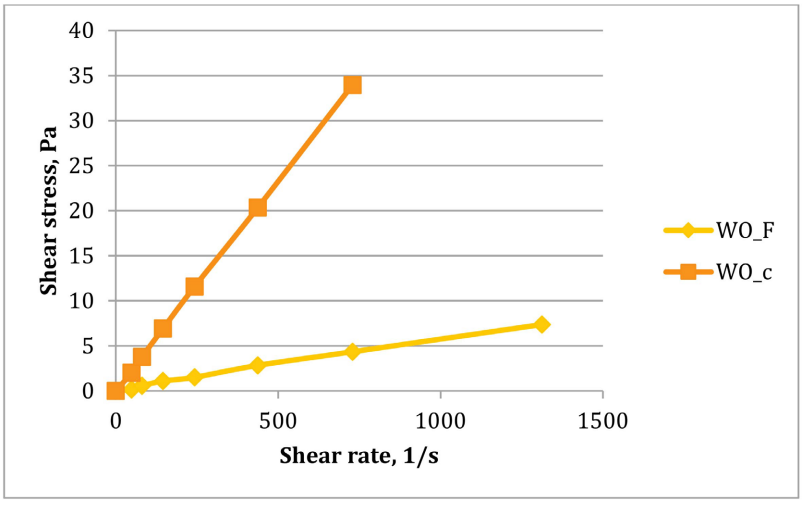


Figure 3. Viscosity of the oil extracted by the Folch method (WO_F) and of the commercial oil (WO_c) at 25˚C.

3.2.3. Humidity Percentage

According to these results (Figure 4), the humidity percentage is: 0.005 × 100/0.262 = 1.91%. The percentage is, therefore, around 2% after purchasing the nuts. This 2% humidity has very little impact on the nut. Indeed, the water in large quantity in a food increases its rate of degradation but here the water is present only in small quantity.

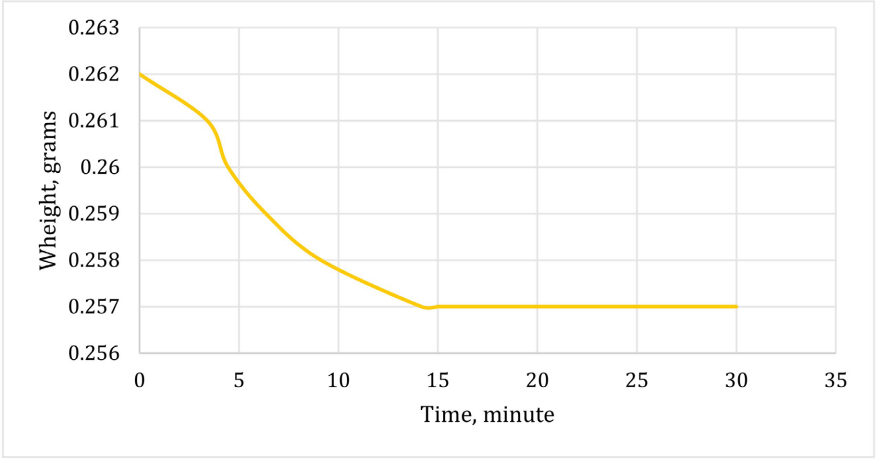


Figure 4. Mass of walnut kernels humidity as a function of time and heated at 100˚C.

3.2.4. Acidity Index

The acidity index [25] is defined by the amount of potash or soda needed, in mg, to neutralize all free fatty acids. This index makes it possible to know if the oil will be of quality regarding the organoleptic and visual criteria for the popular acceptance of oil. One of the main factors that degrade the quality of the oil is the oxidation of triglycerides. This occurs through exposure to light or ambient oxygen, which makes the environment acidic, causing the triglyceride chains to break and release fatty acids.

To measure the degree of oxidation and therefore the amount of free fatty acid, we performed a direct titration of the oil with sodium hydroxide, in the presence of the pH indicator, phenolphthalein (Table 9).

Equation of the reaction of the titration with sulphuric acid:

$$2\mathrm{NaOH} + \mathrm{H_2SO_4} \rightarrow \mathrm{Na_2SO_4} + 2\mathrm{H_2O}$$

$$n_{\mathrm{NaOH}} = 2n_{\mathrm{H_2SO_4}}$$

$$C_{\mathrm{NaOH}} \times V_{\mathrm{NaOH}} \times F_{\mathrm{NaOH}} = 2 \times \left(C_{\mathrm{H_2SO_4}} \times V_{\mathrm{H_2SO_4}} \times F_{\mathrm{H_2SO_4}} \right)$$

$$F_{\mathrm{NaOH}} = \left(2 \times \left(C_{\mathrm{H_2SO_4}} \times V_{\mathrm{H_2SO_4}} \times F_{\mathrm{H_2SO_4}} \right) \right) \Big/ \left(C_{\mathrm{NaOH}} \times V_{\mathrm{NaOH}} \right)$$

$$F_{\mathrm{NaOH}} = \left(2 \times \left(0.1 \times 8.875 \right) \right) \Big/ \left(0.2 \times 10 \right) = 0.8875$$

Equation of the reaction of the titration of free fatty acids by sodium hydroxide:

$$\mathrm{R\text{-}COOH} + \left(\mathrm{Na^+} + \mathrm{OH^-} \right) \rightarrow \left(\mathrm{R\text{-}COO^-} + \mathrm{Na^+} \right) + \mathrm{H_2O}$$

$$n_{\mathrm{NaOH}} = n_{\mathrm{R\text{-}COOH}}$$

$$m_{\mathrm{NaOH}} \times n_{\mathrm{NaOH}} \times M_{\mathrm{NaOH}} = \left(C_{\mathrm{NaOH}} \times V_{\mathrm{NaOH}}\right) \times 39.99$$

$$\mathrm{I.A} = \text{mg of NaOH/1 g of oil}$$

$$\mathrm{I.A} = m_{\mathrm{NaOH}} / m_{\mathrm{oil}}$$

Table 9. Acidity index.

Oil	PITEBA		Soxhlet		Commercial		Folch	
Trial	1	2	1	2	1	2	1	2
Mass of oil (g)	2.17	2.38	2.41	2.66	2.92	2.49	2.18	2.44
Volume of NaOH poured (mL)	0.70	0.70	1.00	0.90	0.60	0.55	1.00	1.1
Real concentration NaOH (mol/L)	0.08875				0.0758			
Mass of NaOH (mg)	2.48	2.48	3.55	3.19	1.82	1.67	3.03	3.33
A.I. (mg/g)	1.09 ± 0.05		1.34 ± 0.17		0.65 ± 0.03		1.38 ± 0.02	

According to the standard [19] for refined fats, this must be less than 0.6 mg of NaOH per gram of oil and for cold-pressed fats and oils around 4.0 mg of NaOH per gram of oil. So, our results agree with the values expected for extra commercial virgin oil and for oil extracted by PITEBA. However, for oil extracted by Soxhlet and by Folch method, this parameter is higher than the optimal. Nevertheless, the results obtained for our oils are roughly similar to each other and slightly higher than that of commercial oil; we can assume that the origin of the nuts is the cause.

3.2.5. Saponification Index

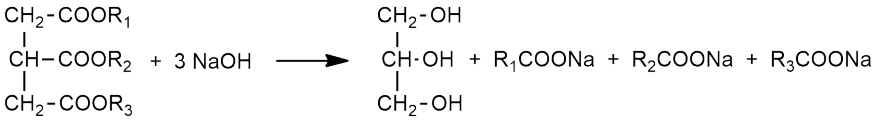
The saponification index [15] is the quantity of sodium hydroxide necessary, in mg, to saponify all the esterified fatty acids and neutralize all the non-esterified fatty acids, that is to say, the free fatty acids that are in the oil sample.

The principle is to react the fatty acids in walnut oil with an excess of sodium hydroxide using the reflux heating system. When the oil and soda solution heats to a boil, the triglycerides split in two, resulting in saponified fatty acids and glycerol. The excess sodium hydroxide present in the solution is then titrated with a hydrochloric acid solution. This titration is indirect, because we determine the amount of excess sodium present in the medium. Then, we determine by difference the amount of potash that was consumed to react with the triglycerides and free fatty acids (**Table 10**).

Table 10. Saponification index.

Oil	PITEBA	Soxhlet	Commercial	Folch
Mass of oil (g)	0.4839	0.4974	0.4856	0.3436
Volume of NaOH poured (mL)	30	34	30	29
Volume of HCl poured (mL)	18.00	21.75	18.4	19.5
S.I. (mg/g)	188	163	192	122

Equation of the saponification reaction:



Equation of the NaOH assay reaction:

$$\mathrm{HCl} + \mathrm{NaOH} \rightarrow \mathrm{NaCl} + \mathrm{H_2O}$$

$$n_{\mathrm{NaOH}} = n_{\mathrm{HCl}}$$

$$n_{\mathrm{HCl}} = C_{\mathrm{HCl}} \times V_{\mathrm{HCl}}$$

$$m_{\mathrm{NaOH\ final}} = C_{\mathrm{HCl}} \times V_{\mathrm{HCl}} \times M_{\mathrm{KOH}} = 0.5(\mathrm{mol/L}) \times V_{\mathrm{HCl}}(\mathrm{L}) \times 56(\mathrm{g/mol})$$

$$m_{\mathrm{NaOH\ final}} = C_{\mathrm{NaOH}} \times V_{\mathrm{NaOH}} \times M_{\mathrm{KOH}} = 0.362(\mathrm{mol/L}) \times V_{\mathrm{NaOH}}(\mathrm{L}) \times 56(\mathrm{g/mol})$$

$$\mathrm{I.S} = \mathrm{mg\ of\ NaOH/1\ g\ of\ oil}$$

$$\mathrm{I.S} = m_{\mathrm{NaOH\ consumed}} / m_{\mathrm{oil}} = (m_{\mathrm{NaOH\ initial}} - m_{\mathrm{NaOH\ final}}) / m_{\mathrm{oil}}$$

According to [26] on the determination of the saponification index , the latter is around 187 to 198 mg KOH / g of oil for walnut oil. The results found for extraction with PITEBA and for commercial oil are closer to the expected result than for the other two oils, but slightly different, perhaps due to the origin of their raw material. The results for Folch and Soxhlet have a lower index. This can be explained by the extraction method using solvents which led to the increased content of minor compounds.

3.2.6. Iodine Index

The iodine index [25] is the mass of iodine, in g, which reacts in 100 g of oil. This index reflexes the amount of unsaturation presents in walnut oil. An iodine-containing reagent is added to the walnut oil solution, which binds to the double bonds of triglycerides. Thereafter, an indirect titration of the excess iodine is carried out with a sodium thiosulfate solution to determine the amount of iodine fixed to the unsaturated acyl chains. During the assay there are two phases, an organic and an aqueous. The organic phase contains the triglyceride molecules and the iodine linked to them. During the assay, the sodium thiosulfate molecules will react with the iodide ions, which are present in excess in the aqueous phase. The solution will then turn brown, yellow and finally colourless. Then, a solution of starch and chloroform was added and a blue complex is obtained, which makes it possible to better highlight the presence of iodine. The reaction that occurs is as follows involves a blue complex formed by amylose, the linear part of starch, with the iodine molecules, which inserts in the coiled structure of the polysaccharide (**Table 11**).

Redox reaction of iodine with sodium thiosulfate solution:

$$2\mathrm{S_2O_3^{2-}} + \mathrm{I_2} \rightarrow \mathrm{S_4O_6^{2-}} + 2\mathrm{I^-}$$

Balance reaction of iodine with sodium thiosulfate solution:

$$2Na_2S_2O_3 + I_2 \rightarrow 2NaI + Na_2S_4O_6$$

The calculation used to find the iodine index (I.I), was as follows [25]:

$$I.I = 1.269 \times V_{Na_2S_4O_6} \times f / m_{huile}$$

Table 11. Iodine index.

Oil	PITEBA		Soxhlet		Commercial		Folch	
Trial	1	2	1	2	1	2	1	2
Mass of oil weighed (g)	0.156	0.150	0.156	0.160	0.149	0.136	0.154	0.153
Volume of sodium thiosulfate poured (mL)	12.50	12.00	12.00	12.55	10.00	9.75	11.40	11.40
Iodine index (g I_2/100 g of oil)	102.0	101.6	97.6	99.5	85.2	92.0	94.0	94.6
	101.8		98.6		88.6		94.3	

The iodine index of each oil is different. This can be explained by the different types of extraction used which have an impact on the final composition of the oils as mentioned above. Finally, for commercial oil, we observe that it has the lowest index. Presumably, it is due to its provenance. Furthermore, not knowing the extraction procedure and the process after extraction for this oil, we can assume that there is a difference between this procedure and that of our oil extracted by PITEBA.

3.2.7. Peroxide Index

This index measures active oxygen, *i.e.*, the amount of free radicals present in the oil. This phenomenon is due to the oxidation of triglycerides by air, which forms hydroperoxides (R-OOH), which then transform into free radical form. To do this, the active oxygen is reacted with potassium iodide and a release of I_2 is obtained. This specie is then titrated with a sodium thiosulfate solution (**Table 12**).

Table 12. Peroxide index.

Oil	PITEBA		Soxhlet		Commercial		Folch	
Trial	1	2	1	2	1	2	1	2
Oil mass (g)	2.32	2.36	2.55	2.55	2.53	2.58	2.36	2.30
Volume of sodium thiosulfate poured (mL)	6.2	6.15	3.5	3.5	2.8	2.75	5.7	5.6
Peroxide index (milliequivalents of active oxygen/Kg of oil)	25.82	25.25	12.96	12.94	10.27	9.88	23.29	23.45
	25.54		12.95		10.08		23.37	

The calculation I used to find the peroxide index (P.I.), is also described in reference [25] and was determined as follows:

$$P.I = \left(10 \times \left(V_2 - V_1\right)\right) / m_{oil}$$

V_2 = volume of sodium thiosulfate for the test in L;

V_1 = Volume of sodium thiosulfate poured for the control in L = 0.0002 L.

The oils extracted by PITEBA and Folch have more active oxygen than commercial oil and that extracted by Soxhlet. There are more free radicals in PITEBA and Folch oil, and the standard indicates a maximum peroxide index of 10 milliequivalents of active oxygen per kilogram of oil for refined oils and 15 milliequivalents of active oxygen per kilogram of oil for oils extracted by cold pressing. The first index concerns the oils extracted by Folch and Soxhlet and neither of these two oils meets the standard. Regarding the second index, only commercial oil meets the standard, indeed, the oil extracted by PITEBA has an index that is above standard values. The presence of oxidized molecules can be explained by the extraction process, which requires heating the press to extract the oil. This process accelerates the oxidation of the oil.

3.2.8. Mineral Analysis

The standard regulation [19] only mentions iron and copper contents. Regarding copper, the standard stipulates 0.1 mg/kg or 0.1 mg/L for refined oils and 0.4 mg/kg or 0.4 mg/L for oils extracted by cold pressing, therefore, only commercial oil and extracted oil by PITEBA meet the standard. Regarding iron, the standard stipulates 1.5 mg/kg or 1.5 mg/L for refined oils and 5.0 mg/kg or 5.0 mg/L for oils extracted by cold pressing, so none of the oils meet the standard. The large amount of iron and zinc for PITEBA is probably due to the extraction where the oil is in direct contact with the press consisting of metals. The higher presence of iron is therefore, certainly due to "pollution" by the material used. In none of the processes is the iron content minimal; each process has an impact on this content. The same applies to the iron content of commercial oil (**Table 13**).

Table 13. Mineral analysis in mg/L.

Mineral	PITEBA	Soxhlet	Commercial	Folch
Pb^{2+}	<0.01	<0.01	<0.01	<0.01
Cu^{2+}	0.43	0.27	0.415	0.098
Zn^{2+}	8.95	1.26	2.131	3.384
Fe^{n+}	36.6	6.63	25.4	28.047
Ca^{2+}	8.1	10.1	6.53	3.47
Mg^{2+}	3.35	13.3	4.57	3.38

3.2.9. UV-Visible Spectroscopy Analysis

This analysis makes it possible to compare the oxidation between the different oils. The oil samples containing a high amount of oxidized molecules, will show high UV absorbance. Oxidized compounds that are created from linoleic and linolenic acids absorb in wavelengths between 232 and 270 nm. It is for this reason that we analysed the samples on these two wavelengths (**Table 14**).

Table 14. UV-visible spectroscopic analysis.

Oil	PITEBA	Soxhlet	Commercial	Folch
232 nm	0.1297	0.3351	0.2087	0.6614
270 nm	0.0151	0.0391	0.0273	0.3096

It can be seen that for the two wavelengths, the oil, which has the most oxidized compounds, is the oil extracted by Folch method. The amount of solvent used to extract the walnut oil is very large and must have led to side reactions creating oxidized compounds present in the oil that could not be removed. Regarding the other three oils, we find, in the order from the least oxidized to the most oxidized, the oil extracted by PITEBA, the commercial oil and then the oil extracted by Soxhlet. This difference is due to the type of extraction and processing that the oils had. In addition, commercial oil has not been analysed at the same time after production as our oils. In fact, three months passed before the analyses, which can explain the slightly higher results than PITEBA.

3.2.10. Infrared Spectroscopy Analysis

Infrared spectroscopy [27] involves subjecting walnut oil to an infrared ray. The molecules of the walnut oil will absorb infrared radiation and will vibrate differently for each atomic group having characteristic bonds and atoms. This analysis was carried out by an infrared device, as described in Material and Methods.

We observe on the infrared spectroscopy (**Figure 5** and **Figure 6**) that the curves corresponding to the 4 oil samples analysed are identical. Only in the oil extracted by Folch is there an additional peak at 757 nm, which corresponds to the valence vibration of the C-H group of aromatic bonds. Regarding the peaks common to the three oils, the peaks between 3009 and 2850 cm^{-1} correspond to the valence vibration of the HC=CH group, which represents the unsaturation's on the triglyceride chains. The peaks around 1743 cm^{-1} correspond to the valence vibration of the C=O group of carboxylic acids. This evidences the presence of oxidized molecules in the oil. The peaks around 1462 cm^{-1} correspond to the valence vibration of the $-CH_3$ group in the carbon chains of the triglycerides. The peaks between 1300 and 1100 cm^{-1} correspond to the valence vibration of the C-O group of the esters. Finally, the peaks around 722 cm^{-1} correspond to the valence vibration of the atomic group $-CH_2$ of vinyl polymers. These represent the triglycerides of the oil.

3.3. CPG/SM Fatty Acids Analysis

This analysis allows characterization of the type and quantity of fatty acids of the oil. Regarding walnut oil, it detects the presence of polyunsaturated fatty acids, which the field of nutrition praises. This analysis is possible because beforehand we reduced the triglycerides of the oil to fatty acid. Triglycerides are non-volatile and therefore impossible to analyse by GC because the technique can only analyse volatile molecules for a certain temperature. For GC, the molecular weight of the analytes must therefore be less than 500 g/mol. It is for this reason that we carry out a derivatization of walnut oil. The method used is taken from [28].

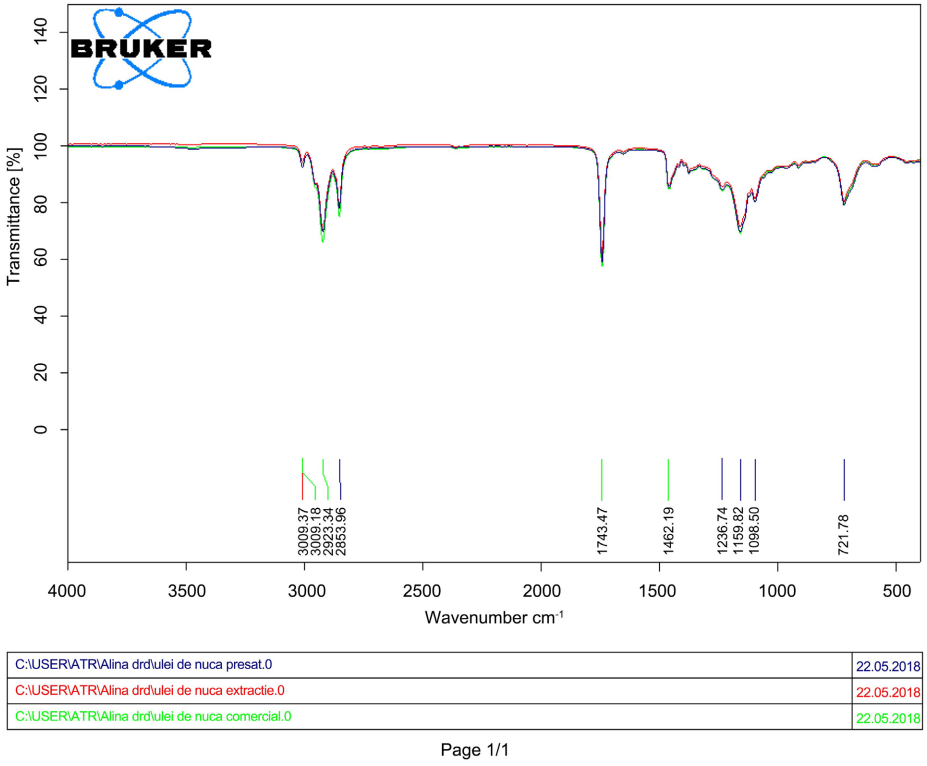


Figure 5. Infrared spectra of PITEB, Soxhlet and commercial oil (Blue curve: oil extracted by PTEBA, Red curve: Oil extracted by Soxhlet, Green curve: extra commercial virgin oil).

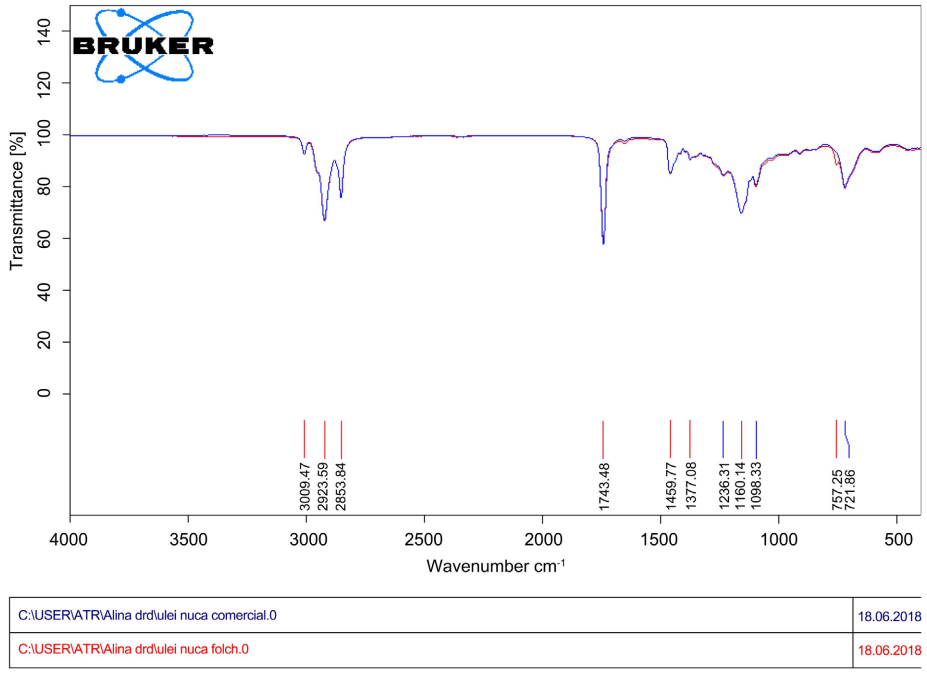


Figure 6. Infrared spectra of Folch and commercial oil (Blue curve: Commercial oil, Red curve: oil extracted by Folch).

To carry out this analysis, we first separated the fatty acids from their glycerol through a derivatization procedure.

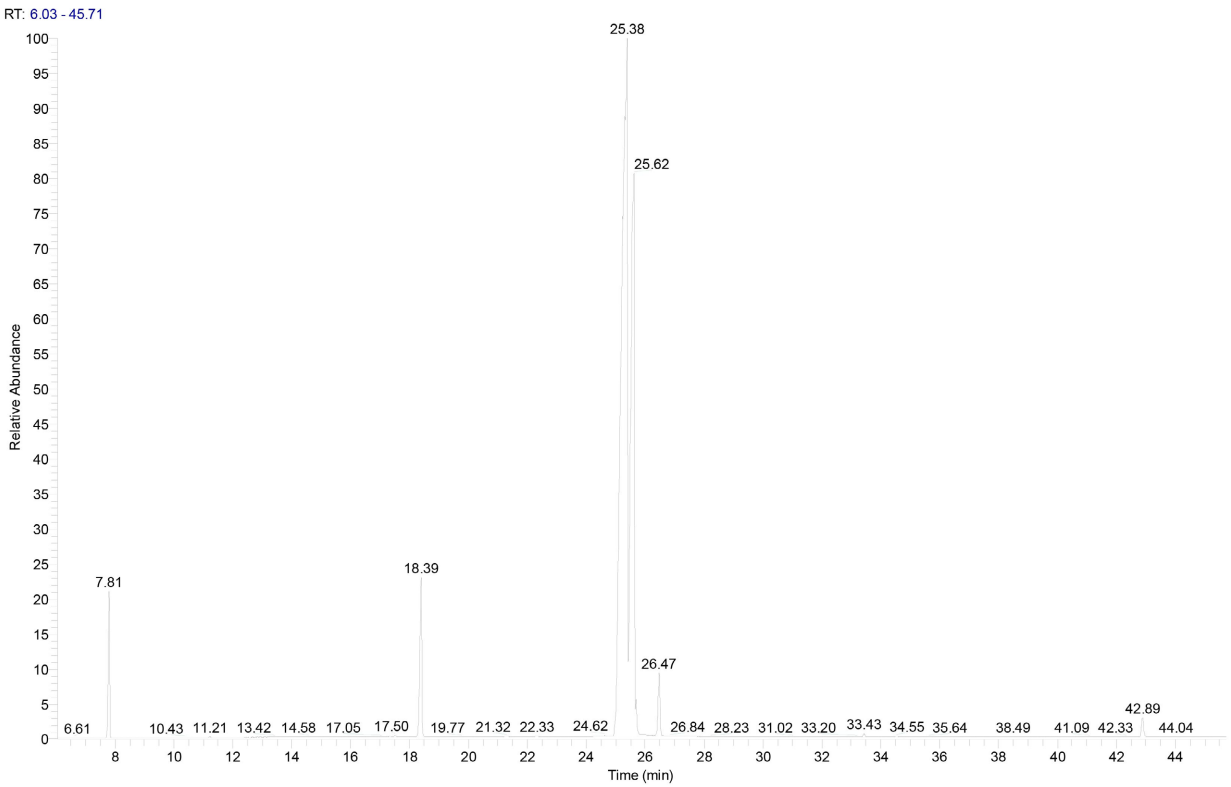
Gas chromatography with mass spectroscopy (GC/MS) makes possible to know the composition and the quantity of the molecules of a solution by separating its components, here the fatty acids of the nut oil.

The separation of fatty acids is based on the difference in affinity of these compounds for the mobile phase and for the stationary phase. The mixture to be analysed is vaporized and then transported through a column containing a liquid or solid substance, which constitutes the stationary phase. Transport is carried out using an inert gas, called "carrier gas", which constitutes the mobile phase.

The more affinity the molecule has for the stationary phase, the less the carrier gas entrains it and therefore the more it is retained on the column. For the analysis, we choose an apolar column, the polar analytes come out first then the slightly less apolar analytes and finally the apolar ones. The higher the temperature of the carrier gas, the more the mobile phase entrains. The analytes separate and then leave the column one after the other. The time between the time of injection and that of column leaving an analyte is called "retention time". These retention times are represented on a graph by peaks with a width and a height specific to each fatty acid (**Figures 7-13**).

3.3.1. Qualitative Analysis

The chromatograms of the fatty acid methyl ester samples of the oils are in Annex 6. A technical problem arose before we had time to analyse the fatty acid sample of the oil extracted by Folch, so we will not report the results for this oil. In addition, during the analysis, contrary to the theoretical data, the presence of linolenic fatty acids was not confirmed. The presence of the other main fatty acids has been confirmed.



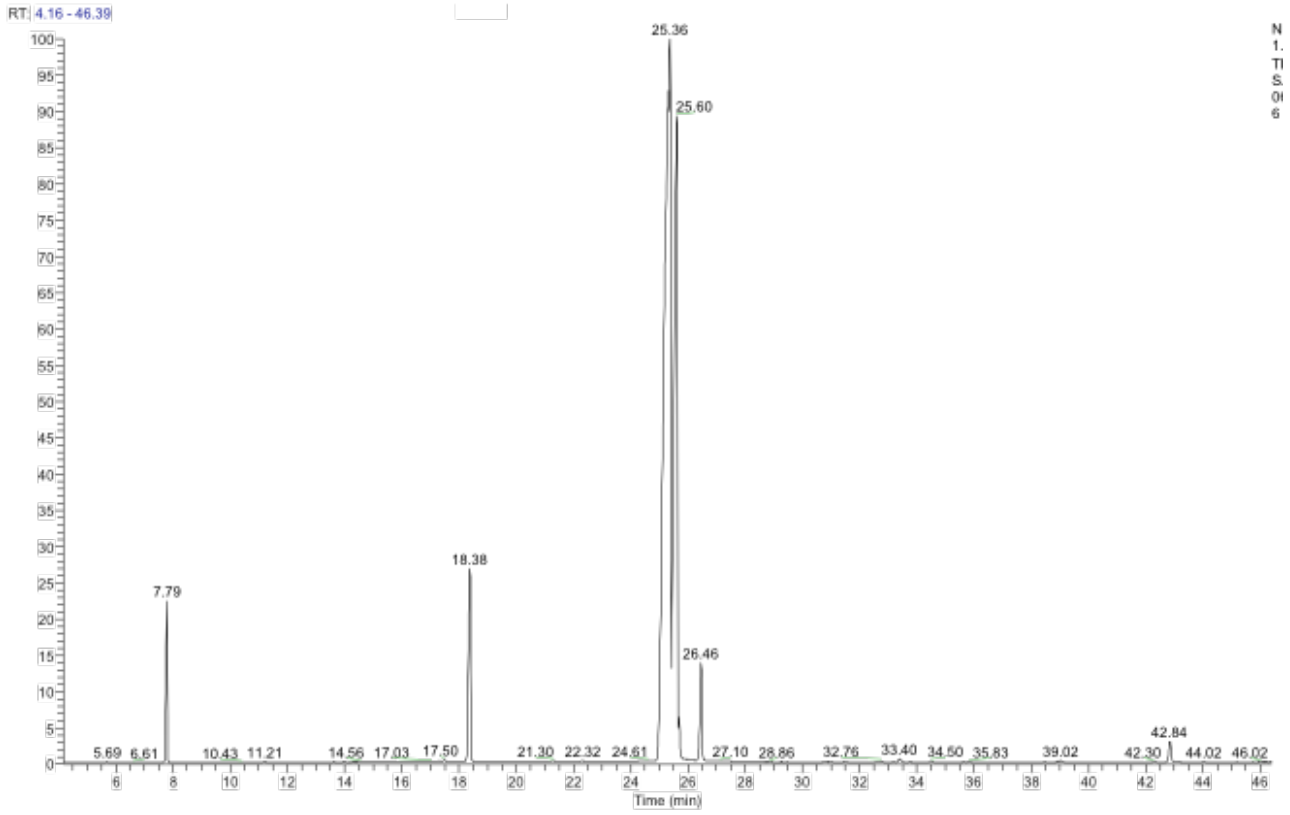


Figure 7. Chromatograms of the fatty acid sample of PITEBA (upper panel) and commercial (lower panel).

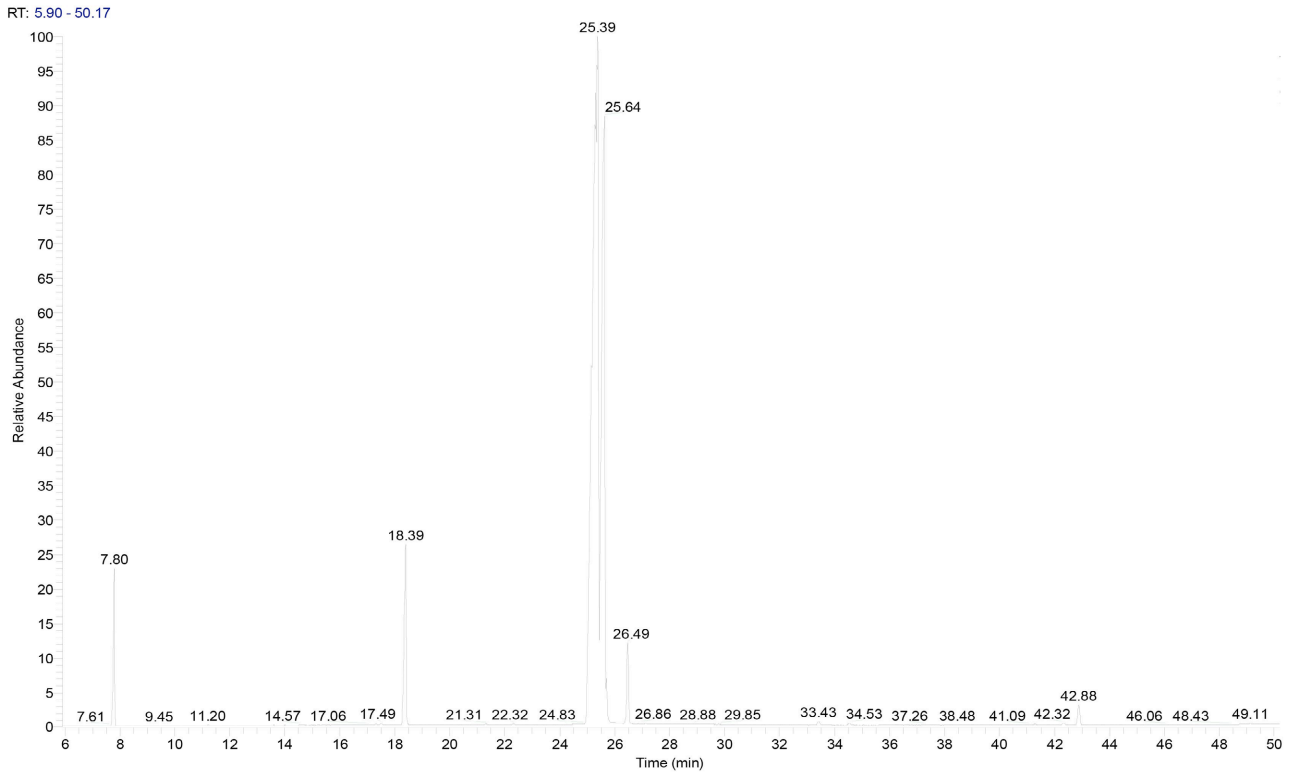


Figure 8. Mass spectrum of Soxhlet oil.

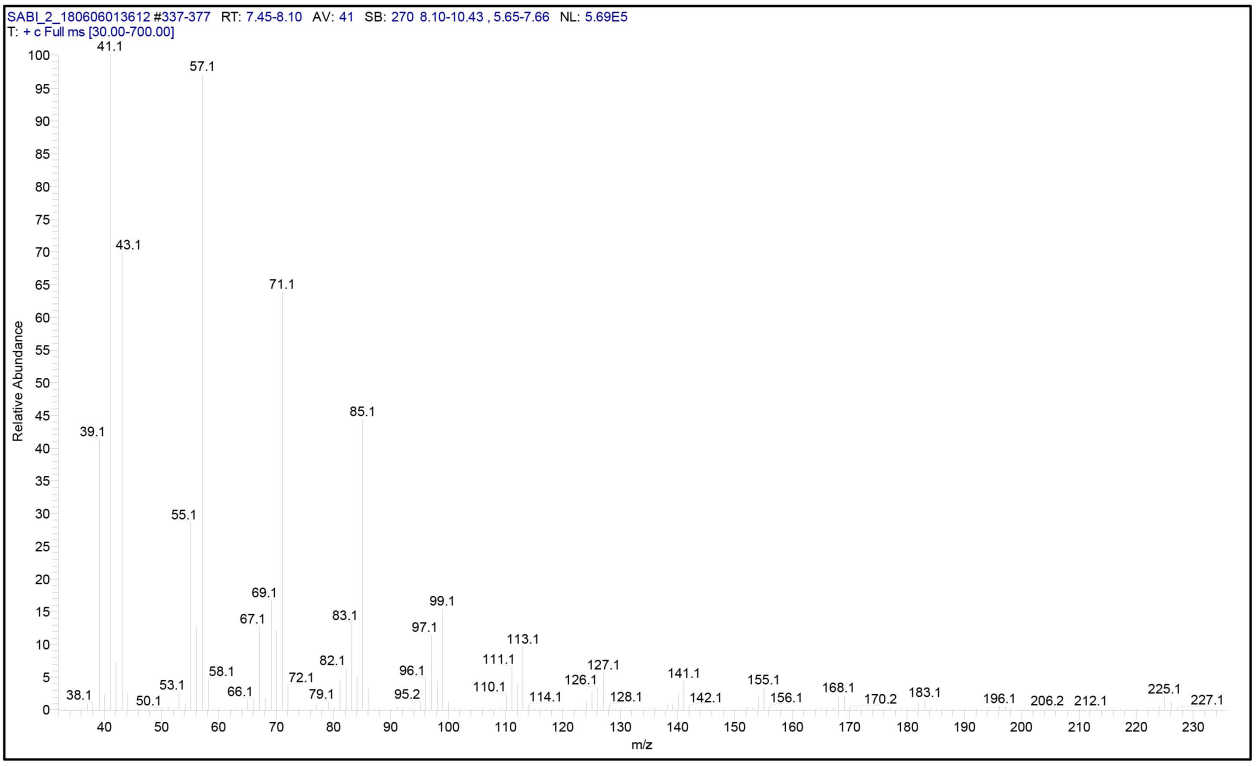


Figure 9. Mass spectrum of hexadecane (retention time 7.52 min).

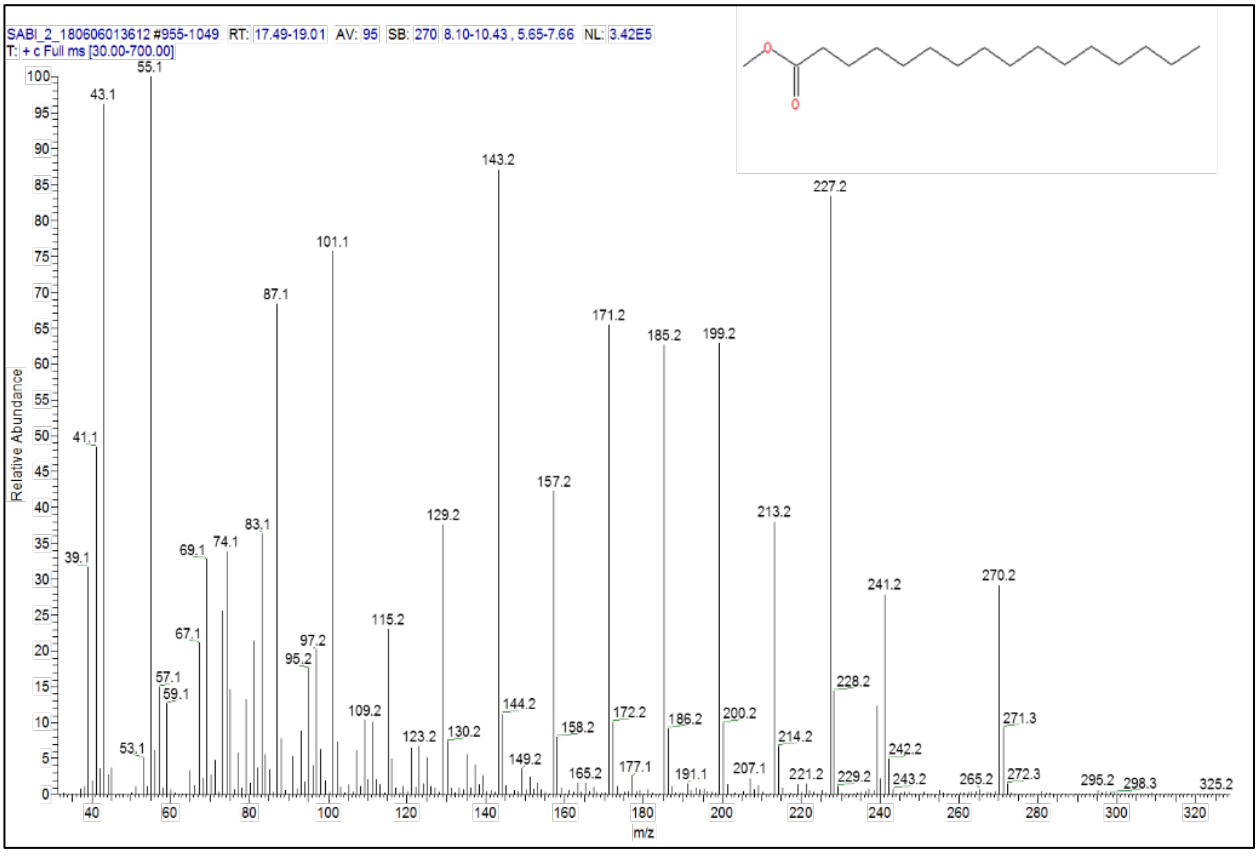


Figure 10. Mass spectrum of palmitic methyl ester (retention time 18.44 min).

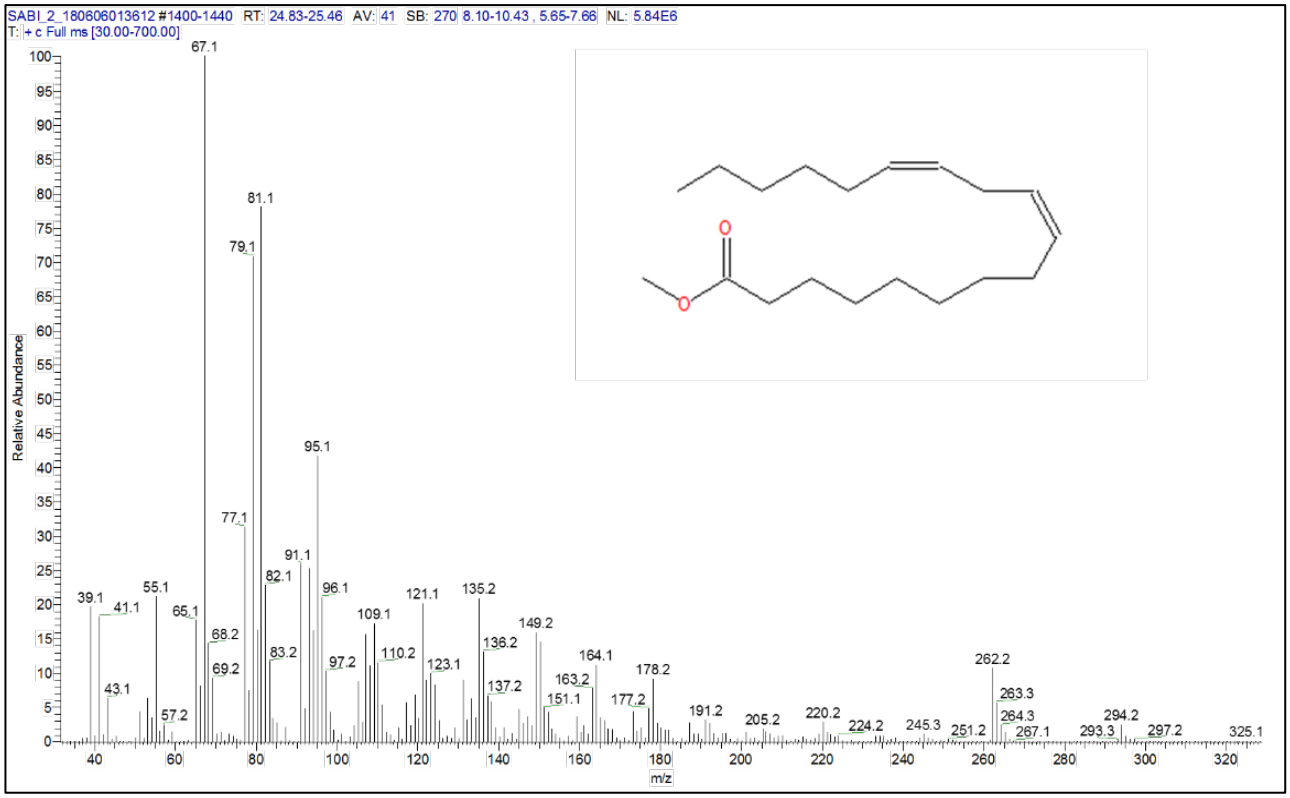


Figure 11. Mass spectrum of linoleic methyl ester (retention time 25.33 min).

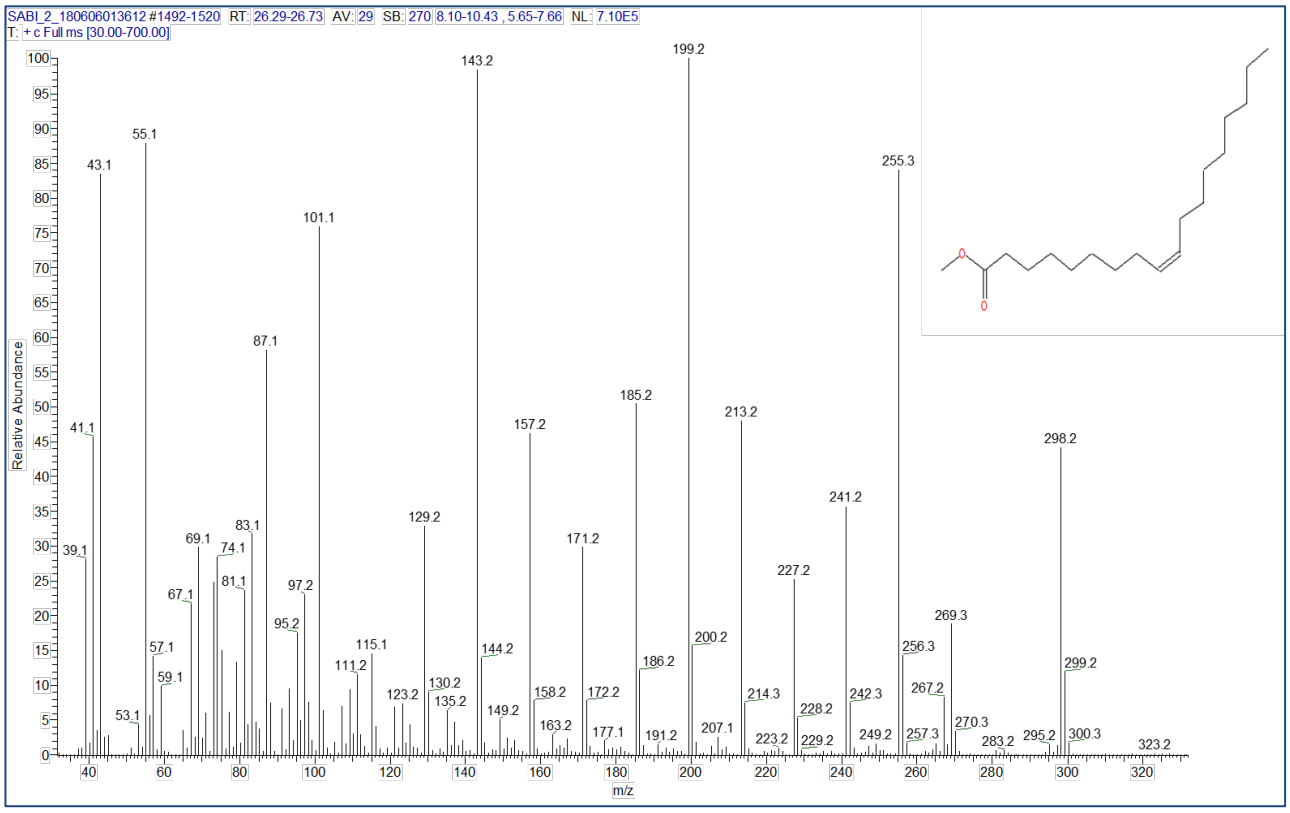


Figure 12. Mass spectrum of oleic methyl ester (retention time 25.66 min).

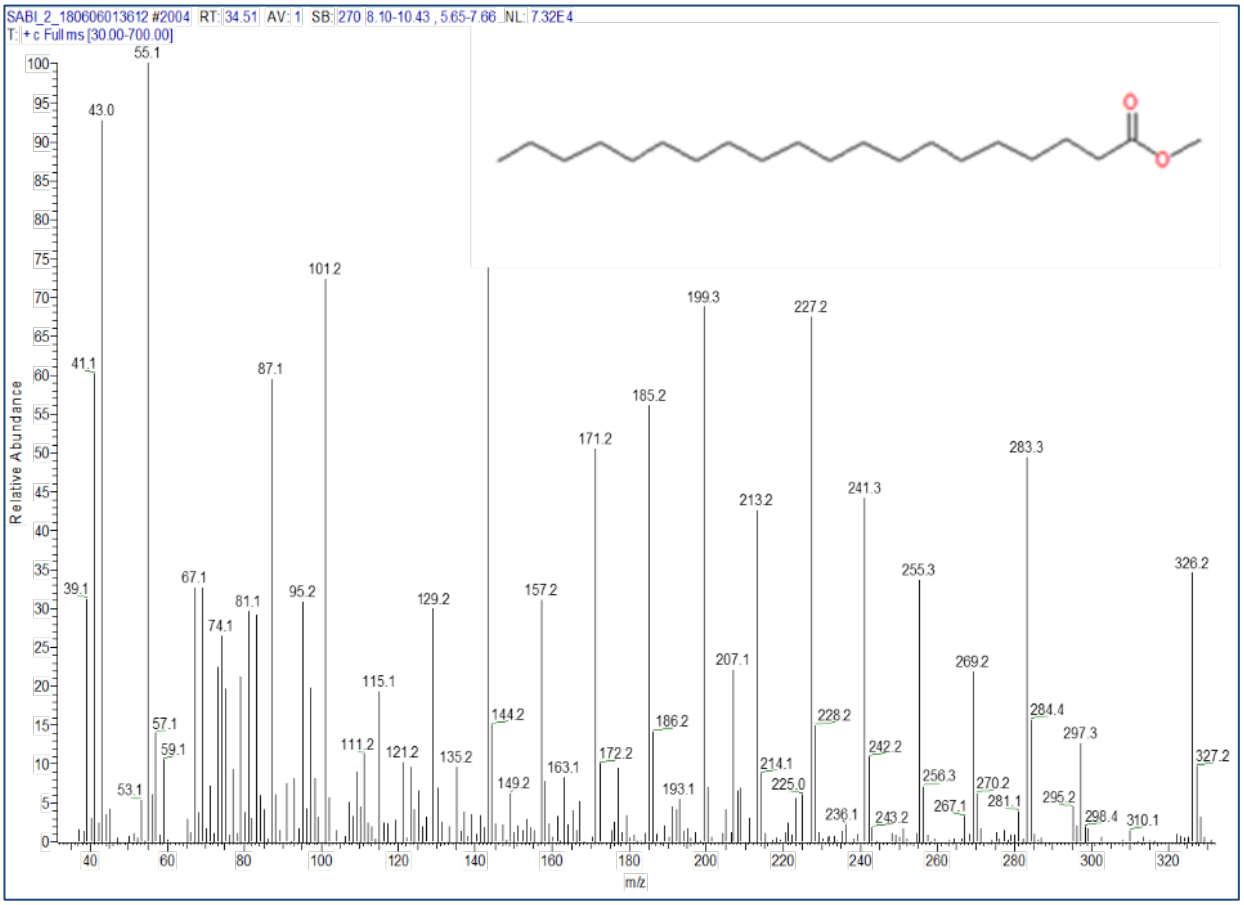


Figure 13. Mass spectrum of eicosenoic methyl ester (retention time 25.54 min).

3.3.2. Fatty Acid Components

This analysis was carried out using calibration curves with a concentration range of 0.05 to 3 mg/mL. The mass spectra of the fatty acids present in the oil, made it possible to make calibration curves in order to know the amount of fatty acid present in the oil (Figure 14, Table 15).

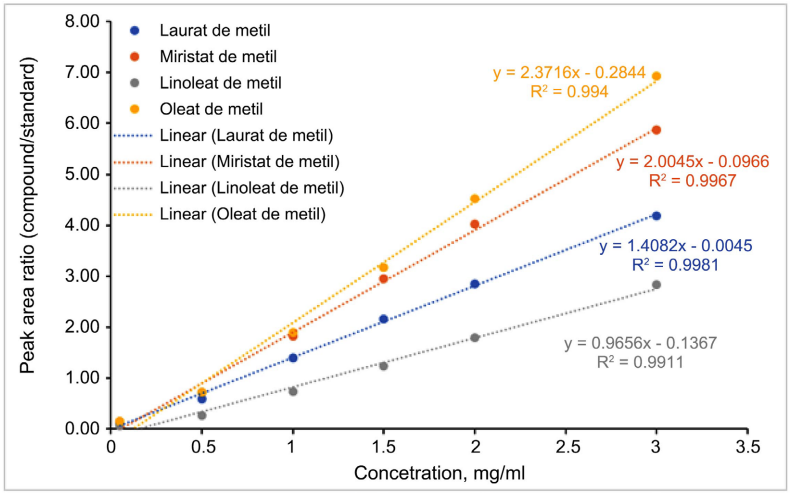


Figure 14. Calibration curve for the quantitative analysis of fatty acids in oils.

Table 15. Quantitative results of fatty acids present in walnut oil.

	Standard	C16:0	C18:2	C18:1	C18:0	C20:0
	A_7.81	A_18.39	A_25.38	A_25.62	A_26.47	A_42.89
PITEBA	1.36E + 08	218415122	2695391777	1267026548	84146855	30665589
Peak area ratio		1.61	19.84	9.32	0.62	0.23
Concentration (mg/mL)			103.42	20.26		
% Fatty acid		5.08	62.75	29.50	1.96	0.71
Commercial	1.46E + 08	275622258	2959325979	1473927879	126795816	32826282
Peak area ratio		1.89	20.25	10.09	0.87	0.22
Concentration (mg/mL)			105.58	21.87		
% Fatty Acid		5.66	60.79	30.27	2.60	0.67
Soxhlet	1.43E + 08	254380125	2901722045	1493472314	106115216	31519787
Peak area ratio		1.78	20.30	10.45	0.74	0.22
Concentration (mg/mL)			105.80	22.62		
% Fatty Acid		5.31	60.61	31.20	2.22	0.66

The percentage of each fatty acid in our oils is about the same as the percentage of commercial oil fatty acids, we observe a high content of polyunsaturated fatty acids (~63%) and monounsaturated fatty acids (~30%) and a very low level of saturated fatty acid (~7%). From the label of extra commercial virgin walnut oil and from the referent results in Appendix 3, we can conclude that our results are consistent. However, compared to the theoretical data, our nut oils from Romanian nuts do not have the presence of linolenic acid or these acids are in a trace state, which makes them invisible for GC/MS.

4. Conclusion

During this study, we extracted the oil from the walnut kernel using different methods, and then characterized these oils according to the extraction process. Finally, the oils extracted by solvent, the Soxhlet and Folch method, are slightly different in terms of their characteristic than the cold extracted oils. Solvent extraction increases the amount of minor components in the oil, which changes its characteristics. Considering that walnut oil is mainly used in the field of nutrition and the food industry, it is therefore important to obtain quality oil and therefore an oil preferably extracted by cold pressing. Our results do not all meet the standards, especially concerning the iron level. Nevertheless, we have obtained good results concerning the walnut oil extracted by PITEBA. Each extraction method has an influence on the quality of the oil. But none of them seems to lead to the optimum oil in terms of its physical, physico-chemical and nutritional characteristics. Regarding its use, walnut oil is presented in food and nutrition in different forms as we have seen. Apart from that, studies are underway on its possible use in the field of biodiesels ad biomedicine, especially as a solution to Alzheimer's

disease. We can assume that in a few years walnut oil could be used in the latter field and thus respond to larger problems.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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